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Bioactivity, chemical profiling and 16S rRNA based phylogeny of *Pseudoalteromonas* strains collected on a global research cruise

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ABSTRACT

One hundred one antibacterial *Pseudoalteromonas* strains that inhibited growth of a *Vibrio anguillarum* test strain were collected on a global research cruise (Galathea 3), and 51 of the strains repeatedly demonstrated antibacterial activity. Here, we profile secondary metabolites of these strains to determine if particular compounds serve as strain or species markers and to determine if the secondary metabolite profile of one strain represents the bioactivity of the entire species. 16S rRNA gene similarity divided the strains into two primary groups: One group (51 strains) consisted of bacteria which retained antibacterial activity, 48 of which were pigmented, and another group (50 strains) of bacteria which lost antibacterial activity upon sub-culturing, two of which were pigmented. The group that retained antibacterial activity consisted of six clusters in which strains were identified as *Pseudoalteromonas luteoviolacea, Pseudoalteromonas aurantia, Pseudoalteromonas phenolica, Pseudoalteromonas ruthenica, Pseudoalteromonas rubra* and *Pseudoalteromonas piscicida*. HPLC-UV/VIS analyses identified key peaks, such as violacein in *P. luteoviolacea*. Some compounds, such as a novel bromoalterochromide were detected in several species. HPLC-UV/VIS detected systematic intra-species differences for some groups and testing several strains of a species was required to determine these differences. The majority of non-antibacterial, non-pigmented strains were identified as *Pseudoalteromonas agarivorans* and HPLC-UV/VIS did not further differentiate this group. *Pseudoalteromonas* retaining antibacterial were more likely to originate from biotic or abiotic surfaces in contrast to planktonic strains. Hence, the pigmented, antibacterial *Pseudoalteromonas* have a niche specificity, and sampling from marine biofilm environments is a strategy for isolating novel marine bacteria that produce antibacterial compounds.

Words: 252

KEYWORDS: *Pseudoalteromonas*, antibacterial activity, secondary metabolites, bioprospecting, Galathea 3

INTRODUCTION

Compounds of relevance for the pharmaceutical and biotechnology industries are produced by marine microorganisms (Burgess et al., 1999) and it has been suggested that some compounds of pharmacological interest previously attributed to macroorganisms may in fact be of microbial origin (Bewley and Faulkner, 1998;Simmons et al., 2008;Sudek et al., 2006). The emergence of multiresistant pathogenic bacterial strains and the failure of combinatorial and diversity oriented chemistry to adequately supply the drug discovery pipeline (Newman, 2008) has re-invigorated natural product chemistry as a path for discovery and development of new antibiotics. With this in mind, we isolated marine bacteria with antibacterial activity during the Danish Galathea 3 marine research expedition (Gram et al., 2010). The antibacterial strains were tentatively identified using 16S rRNA-similarity and one of the major groups of isolated bacteria was identified as *Pseudoalteromonas* (Gram et al., 2010).

The genus *Pseudoalteromonas* consists of Gram negative marine bacteria belonging to the γ-proteobacteria and are present globally in marine waters where they constitute 0.5 to 6% of the total bacterioplankton (Wietz et al., 2010). They are heterotrophic aerobes, non-fermentative and the cells are motile by one or more polar flagella. The genus divides into two groups; pigmented and non-pigmented species. The pigmented species are often producers of bioactive secondary metabolites (Bowman, 2007) displaying cytotoxic (Zheng et al., 2006), antibacterial (Gauthier, 1976b;Gauthier and Flatau, 1976;Isnansetyo and Kamei, 2003;Jiang et al., 2000;McCarthy et al., 1994), antifungal (Franks et al., 2006;Kalinovskaya et al., 2004) or antifouling (Egan et al., 2001;Holmström et al., 2002) effects. It has been hypothesized that bioactive *Pseudoalteromonas* are primarily associated with higher organisms (Holmström and Kjelleberg, 1999), suggesting an ecological role in which some bioactive species might play an active part in host defense against pathogens and fouling organisms (Holmström et al., 1996;Armstrong et al., 2001;Egan et al., 2008).

A link between surface colonization and antibacterial activity has not been experimentally verified, although several studies have successfully isolated epiphytic bacteria with antibacterial activity from algae and other marine organisms (Armstrong et al., 2001;Boyd et al., 1999;James et al., 1996;Penesyan et al., 2009). The group of non-pigmented species have highly similar 16S rRNA gene sequences (Ivanova et al., 2004) and is rarely inhibitory against other microorganisms, although e.g. *P. haloplanktis* strain INH produces isovaleric acid and 2-methylbutyric acid showing a broad spectrum of bacterial inhibition (Hayashida-Soiza et al., 2008).

Phylogeny and differentiation of bacterial species rely heavily on 16S rRNA gene similarity (Stackebrandt et al., 2002) however, 16S rRNA gene similarity does not provide sufficient differentiation below species level (Fox et al., 1992). Comparison of secondary metabolite production has supported species delineation within the Actinomycete genus *Salinispora*, where strains with high 16S rRNA similarity were shown to belong to distinct species each with different specific metabolite profiles (Jensen et al., 2007). In mycology, comparison of chemical profiles (e.g. TLC, direct-infusion mass spectrometry, and HPLC with various detectors such as UV/VIS and/or mass spectrometry) of secondary metabolites has been widely used to identify and differentiate filamentous fungi (chemotaxonomy) also at sub-species level (Frisvad et al., 2008), and the chemophylogeny correlates well with phylogenetic analysis of sequences of specific housekeeping genes (e.g. chitin synthase, β-tubulin and calmodulin) (Geiser et al., 2007). Since several *Pseudoalteromonas* species produce a range of secondary metabolites, we hypothesized that chemical profiling and specific marker compounds could be indicative of bioactive potential and at the same time be useful in species identification or differentiation (Jensen et al., 2007).

The aim of the present study was to profile the secondary metabolites of these strains to determine if particular compounds serve as markers of strains or species with antibacterial activity, and to determine if several strains of each species must be tested to assess the full bioactivity potential. As

part of this we provide accurate identification and phylogeny of these organisms by detailed 16S rRNA gene sequence comparative analysis. Since the bacteria were isolated from different sample types, our collection also allows us to address aspects of *Pseudoalteromonas* ecology such as the possible link between surface or planktonic lifestyle and antibacterial activity.

MATERIALS AND METHODS

Strain isolation. Approx. 500 marine bacterial strains with antagonist activity against *V. anguillarum* strain 90-11-287 (Skov et al., 1995) were isolated during the Danish Galathea 3 research expedition (Gram et al., 2010). One hundred and one of these strains tentatively identified as *Pseudoalteromonas* species were included in the present study. *V. anguillarum* 90-11-287 was used as target strain since the expedition ship was not equipped to handle e.g. potential human pathogens, and this *Vibrio* strain is in our experience very sensitive to antibacterial compounds from other marine bacteria (Hjelm et al., 2004).

Growth media and culture conditions. *Pseudoalteromonas* strains were grown in marine broth (MB) 2216 (Difco, Detroit, USA) and on marine agar (MA) 2216 (Difco, Detroit, USA) prepared in accordance with the manufacturer's instructions. Broth cultures were incubated under stagnant conditions at 25ºC. Pigment production was determined by visual inspection of 48 hour old culture broths (MB) and colonies grown on MA for 24 to 48 hours.

Antibacterial activity. Instant Ocean (IO) bioassay agar plates were prepared as described by Hjelm *et al.* (Hjelm et al., 2004). Ten g/l agar, 3.3 g/l casamino acids (Difco 223050, Detroit, USA) and 30 g/l Instant Ocean aquatic salts (Instant Ocean® Aquarium systems Inc., Sarrebourg, France) were added to distilled water and autoclaved. Glucose (0.4%) and 10 µl/ml of *V. anguillarum* overnight culture was added to the cooled (44ºC) IO and plates poured. The plates were allowed to dry for 15 minutes and if used for well diffusion agar assays (WDAA), wells (diameter 6 mm) were punched. The inhibitory activity of live *Pseudoalteromonas* bacterial cells was tested by spotting 48 hour old MA grown colonies on freshly prepared IO-agar plates containing *V. anguillarum*. Plates were incubated at 25ºC and inspected for clearing zones in the growth of *V. anguillarum* after 24h.

Cell-free supernatants were prepared to test for the presence of water soluble antibacterial compounds secreted to the broth and ethyl acetate extracts were prepared to test for production of non-polar antibacterial compounds. Each strain was grown in 20 ml of MB for 48 h. A 1.5 ml sample was withdrawn for 0.2 µm filtering and subsequently the remainder of the culture was extracted with an equal volume of ethyl acetate. The ethyl acetate fraction was transferred to a new vessel, evaporated to dryness and redissolved in 2 × 0.5 ml ethyl acetate. The 1.5 ml cell-free sterile filtered supernatant and the ethyl acetate extracts were stored at -20ºC until tested in the WDAA (50 µl sample per well) based on IO-agar plates containing *V. anguillarum*. Controls (sterile MB and pure ethyl acetate) did not cause any inhibition zones.

The number of antibacterial *Pseudoalteromonas* strains in surface samples (e.g. algae, driftwood, fish and sediment samples) was compared to their numbers in water samples by the Fisher's exact test (Fisher, 1958). A 2 × 2 contingency table was used to test the hypothesis that presumed antibacterial strains with stable antibacterial activity were equally likely to be isolated from water samples and surface samples.

16S rRNA gene sequence analyses. A detailed phylogenetic analysis was performed on 16S rRNA sequences obtained in a previous study (Gram et al., 2010). For the analysis in this study, we conducted a BLAST (http://blast.ncbi.nlm.nih.gov) search against a compilation of *Pseudoalteromonas* type strain sequences retrieved from GenBank (list of type strains obtained from http://www.bacterio.cict.fr) and sequences of the type strains with a BLAST match in our strain collection were included in 16S rRNA sequence analysis to obtain a robust phylogenetic tree. Sequences from two additional *Pseudoalteromonas* strains were included: The genus type strain *P. haloplanktis* and the bioactive *P. tunicata*. *Salinispora arenicola* CNS-205 was used as out-group. The sequences were aligned by the MAFFT online software (http://www.ebi.ac.uk/Tools/mafft/) (Katoh et al., 2002) and curated with the Gblocks software on its least stringent settings

(Castresana, 2000;Talavera and Castresana, 2007). The resulting alignment was processed using the MEGA4 software (Tamura et al., 2007) to create neighbor joining and minimum evolution trees. PhyML 3.0 was used to generate a maximum likelihood tree (Guindon and Gascuel, 2003). Phylogenetic trees were generated under default parameters with 1,000 bootstrap replications for neighbor joining and minimum evolution trees, and 100 bootstrap replications for the maximum likelihood tree. GenBank accession numbers for the *Pseudoalteromonas* strains used in this study are included in supplementary table 1.

HPLC-UV/VIS analysis of secondary metabolites. The strains were grown in static cultures in 10 ml MB for 3 days at 25ºC, and for each species one strain was cultured in triplicate to establish extraction and growth variation. Cultures were extracted with equal volumes of ethyl acetate, centrifuged and the ethyl acetate and evaporated under N_2 to dryness. Samples were re-dissolved in 300 µl acetonitrile-water (1:1 v/v), and filtered through a 13 mm ID PFTE syringe filter. A subsample of 2 µl was then analyzed by reversed phase HPLC on an Agilent 1100 System equipped with a UV/VIS photo diode array detector (DAD, scanning 200-600 nm). Separation was done on a 100 mm × 2 mm i.d., 3 µm Gemini C_6-phenyl column (Phenomenex, Torrance, CA), running at 40°C using a binary linear solvent system of water (A) and acetonitrile (B) (both buffered with 50 µl/l trifluoroacetic acid) at a flow of 300 µl/min. The gradient profile was: t= 0 min, 5% B; t =22 min, 70% B; t=24.5,100% B, t= 27 min, 100% B; t =29 min, B= 5%; holding this for 8 minutes prior to the next injection. The chromatographic profiles were compared, subtracting peaks present in media blank extracts. Samples were analyzed in random order, and 6 of the first extracts were analyzed several times during the sequence to determine any retention time shifts. Cluster analysis was done on a matrix of detected / non-detected peaks (1/0) using NTSYSpc 2.20q (Exeter Software, Setauket, NY). SAHN clustering was used by UPGMA (unweighted pair-group method) and simple distance measurement. Representative extracts were also analyzed by HPLC-UV/VIS-TOFMS in both positive and negative electro spray (Nielsen and Smedsgaard, 2003). Peaks were tentatively identified by UV-

spectra and accurate mass data by matching in Antibase 2009 (35 930 microbial secondary metabolites) (Wiley & Sons, Hoboken, NJ) (Nielsen et al., 2006).

RESULTS

Pigmentation and antibacterial activity. The one hundred and one *Pseudoalteromonas* strains were originally isolated for their ability to inhibit *V. anguillarum* (Gram et al., 2010). However, on re-cultivation and re-testing for the ability to inhibit *V. anguillarum* after storage at -80ºC for several months, only 51 strains retained inhibitory activity (Table 1). These 51 strains were all inhibitory when tested as live cultures in the 'spot test' assay. Twenty of the strains produced water-soluble, diffusible antibacterial substances as indicated by the ability of cell-free sterile filtered supernatant to inhibit growth of *V. anguillarum* in the WDAA. These 20 strains were identified as *P. phenolica* (1 strain), *P. luteoviolacea* (5 strains), *P. rubra* (9 strains), *P. citrea* (1 strain) and *P. aurantia* (4 strains). Ethyl acetate extraction of culture broths resulted in 19 crude extracts which inhibited growth of *v. anguillarum* in the WDAA. Four of these were identified as *P. luteoviolacea* and were the only strains where both cell-free supernatant and crude ethyl acetate extracts inhibited *Vibrio* growth. The remaining 15 inhibitory crude extracts all originated from strains identified as *P. ruthenica.* The crude ethyl acetate extracts of 26 strains were intensely colored, however only some of these extracts inhibited growth of *Vibrio* indicating that the pigments were not universally antibacterial. Cell-free culture supernatants and ethyl acetate crude extracts of strains with no growth inhibition of *V. anguillarum* in the 'spot test' assay were also tested but showed no growth inhibition.

Forty eight of the fifty one antibacterial strains were pigmented, while two (S3431 and S3655) of the fifty non-active strains were pigmented (Table 1). Antibacterial activity was significantly more likely to be produced by pigmented strains as determined by Fisher's exact test (2-tailed *p*-value of 0.0000). In total, 70 strains were isolated from surface swabs and 31 from water samples. Of the surface associated strains 45 remained active in the spot-assay in comparison to 6 of the water sample strains. The Fisher's exact test demonstrated a significant relation between surface association and stable antibacterial activity (2-tailed *p*-value of 0.0000).

Pseudoalteromonas strains were isolated on all parts of the global cruise in both tropical and temperate waters (Figure 1). Our strain collection is not large enough to allow for complete biogeographic analysis but pigmented strains appeared to be more frequent in coastal areas whereas the non-pigmented strains appeared associated with open waters (Figure 1).

16S rRNA gene sequence analyses. We initially performed a BLAST search querying the 16S rRNA gene sequence of each strain against the GenBank database. The results of this analysis were ambiguous, as some sequences returned more than 40 hits all with identical scores in the BLAST results (data not shown), frequently including the sequences of several different *Pseudoalteromonas* species. Therefore, a BLAST analysis was carried out querying the sequences against a complete set of *Pseudoalteromonas* type strains and hence each strain is matched with the best type strain BLAST match (Suppl Table 1).

The 16S rRNA gene sequences were used to cluster the strains by constructing a neighbor-joining tree and branch support was verified by comparison to minimum evolution and maximum likelihood trees (Figure 2). Nodes supported by an 80% bootstrap cut-off were collapsed when 3 or more strains were included in the cluster (Figure 2). An exception was made for clusters VI and VII, which are shown as separate clusters due to obvious differences in phenotype (pigment, bioactivity, secondary metabolite profile). Ninety-nine of the strains fell into one of 8 primary clusters.

Clusters I and III consisted of non-pigmented non-inhibitory strains (Table 1). Cluster I included 38 strains and the type strains of *P. haloplanktis, P. agarivorans*, *P. tetraodonis, P. paragorgicola*, *P. distincta, P. arctica, P. nigrifaciens, P. elyakovii, P. carrageenovora, P. marina* and *P. aliena*. Strain S3431 – a black pigmented strain in cluster I – did show more than 97% similarity even when

compared to the full GenBank database which suggests that S3431 could represent a novel *Pseudoalteromonas* species. Despite the low BLAST similarity score, phylogenetic analysis and tree construction placed strain S3431 in the diverse cluster I (the non-collapsed cluster I is shown in supplementary figure 1). Cluster III contained no type strains which supported the BLAST analysis where the strains in cluster III were 97% similar to the best type strain match (Supplementary table 1).

The remaining six of the eight clusters contained pigmented strains. Pale yellow strains clustered with the type strains of *P. citrea* and *P. aurantia* (cluster II) and four intensely purple strains grouped in cluster V with the type strain of *P. luteoviolacea*. Cluster VI contained nine red-pigmented strains and the type strain of *P. rubra* and cluster VII consisted of 12 intensely yellow strains, 1 pale yellow strain and the *P. flavipulchra, P. maricaloris* and *P. piscicida* type strains. Fifteen strains and their nearest BLAST match, *P. ruthenica*, formed cluster VII. These strains all produced a pale brown pigment. Cluster IV contained six strains and the type strain of *P. phenolica*. Four of the strains in this cluster had *P. phenolica* as their best type strain BLAST match; however strain S1093 had *P. luteoviolacea* as its best match at 98% identity, while *P. rubra* and *P. luteoviolacea* type strains scored identically (97%) as the best matches for S2724. The strains in cluster IV were heterogeneous with respect to pigmentation, some were non-pigmented and others appeared brown.

Profiling of secondary metabolites. The 101 strains and select type strains were separable in discrete groups by HPLC-UV/VIS (Figure 3) and the triplicate profiles from an isolate of each species were very reproducible and could be superimposed on each other (data not shown). All of the 38 strains of the 16S rRNA cluster I fell into group A, in which no UV/VIS peaks were unique compared to the media blanks indicating that no secondary metabolites were produced. This large group also included all nine strains from 16S rRNA cluster III and strains of *P. phenolica* and *P.*

ruthenica less proficient in secondary metabolite production. A summary of the detected compounds is shown in table 2 and the producer organisms are shown by 16S rRNA cluster in table 3.

Based on their production of specific metabolites, the majority of pigmented bacteria formed six main groups not including four *P. rubra* strains (Figure 3). In the pigmented bacteria, a total of 26 distinct peaks were detected and included in the cluster analysis. We identified indolmycin, violacein, and prodigiosin among the significant peaks based on reference standards. Furthermore, 9 peaks could be tentatively identified based on HPLC-UV/VIS-TOFMS results and data in Antibase2009. These nine included two likely novel bromoalterochromides and a brominated indole (Table 2).

Comparing the 16S rRNA gene sequence clusters with the chemical profiling revealed several patterns. Some compounds were exclusively produced by strains belonging to the same cluster whereas other compounds were produced across several strains from different clusters. All strains in cluster II (*P. aurantia / P. citrea*) shared production of compound B (retention time, RT 12.31 min) whereas the production of 4 other compounds F, Q, T and U (RT 15.47, 16.60, 17.96 and 18.28 min), were scattered in the group. This included a novel bromoalterochromide (compound Q, RT 16.60 min) that was also found in cluster VII (*P. flavipulchra/P. piscicida*). Three of the other compounds were identified as quinolins based on distinct UV spectra and accurate mass.

P. luteoviolacea strains (cluster V) shared production of compound D (violacein, RT 14.29) in all four strains and the type strain, but were sub-divided by compound A (indolmycin, RT 11.21) produced by 2 strains and compound Z (pentabromopseudilin, RT 22.65) produced by the 2 other strains and the type strain. This division is visible in figure 4, which shows chromatograms of the 4 strains in cluster V. Interestingly, also two *P. phenolica* strains produced compound Z.

Cluster VII (*P. flavipulchra* and *P. piscicida*) was chemically very homogeneous. All strains except one produced three bromoalterochromides P, Q and R (RT 16.49, 16.60 and 17.10) of which Q and R were novel compounds. In contrast to cluster V and compound D, the production of P, Q and R was not a unique marker for strains of this cluster as P, Q and R were also detected in one strain from cluster II and one strain from cluster VI.

Thirteen of the 15 strains in cluster VIII, identified as *P. ruthenica*, shared a unique chemical profile and produced the compounds H, K and O (RT 15.78, 15.98, 16.30) with characteristic UV spectra. None of these matched compounds in Antibase2009 and potentially constitute novel antibacterials. These compounds were not detected among strains from other clusters, yet they were not suitable as a distinct chemical marker for cluster VIII since no secondary metabolites were detected in the remaining two strains in this cluster or the type strain.

The strains in cluster VI were identified as *P. rubra* and were chemically very heterogeneous. Five of nine strains produced the red pigment prodigiosin (compound M, RT 16.00) which was not detected in strains of other clusters. Additionally, a multitude of known and non-identified compounds were detected in one or more strains in the cluster. In total, 16 compounds were detected within the cluster and 12 of these were unique for this cluster. Only two strains shared an identical production of secondary metabolites, further stressing the chemical diversity among the strains in this cluster.

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DISCUSSION

We demonstrate in this study, in agreement with earlier findings (Bowman, 2007), that species within the *Pseudoalteromonas* genus produce a range of secondary metabolites, some with antibacterial activity. Several species of the genus are intensely pigmented and it is hypothesized that pigmentation co-occur with antibacterial activity (Egan et al., 2002). In our global collection of *Pseudoalteromonas* strains that demonstrated antibacterial activity on initial isolation, strains that were pigmented were significantly more likely to retain antibacterial activity on re-growth than non-pigmented strains. Several intensely coloured organic extracts were not inhibitory against *V. anguillarum* and hence we do not believe that the pigments, in general, are the cause of the antibacterial activity although e.g. the purple pigment violacein is a known antibiotic compound (Lichstein and Vandesand, 1945).

Nearly half of the isolated strains did not retain any antibacterial effect after frozen storage and subculturing despite being isolated on the original plates due to antibacterial activity. The observed loss of antibacterial activity may be due to a requirement for factors specific to local seawater, as initial tests for antibacterial activity were carried out using 50% local seawater (Gram et al., 2010). Furthermore, loss of antibacterial activity may be due to repression or inhibition of gene clusters encoding products that are required for secondary metabolite synthesis (e.g. by catabolite repression). A reduction in antibiotic production when the producer organism is grown in excess of a carbon source is a known phenomenon (Sanchez et al., 2010) and suppression of secondary metabolite production by excess concentrations of other substrate components is demonstrated in *Streptomyces* (Doull and Vining, 1990). This could suggest that culturing the strains under nutrient limited conditions may reestablish production of antibacterial compounds. Also, during the original sampling and screening procedure, the agar plates may have harbored co-cultured microorganisms which potentially induce antibacterial activity as has been demonstrated by Mearns-Spragg et al.

(Mearns-Spragg et al., 1998). Hence, it may be possible to re-induce the antibacterial activity if the right conditions can be created.

Several bioactive *Pseudoalteromonas* have been isolated from higher organisms and it has been hypothesised that antibacterial compounds may play a role in bacterial competition or as protective agents beneficial for the host organism (Holmström and Kjelleberg, 1999). We provide statistical evidence that surface associated presumed antibacterial pseudoalteromonads are significantly more likely to show stable production of antibacterial compounds than *Pseudoalteromonas* species isolated as planktonic cells. This suggests that production of antibacterial compounds may play an important role in the ability of *Pseudoalteromonas* strains to colonize and persist on surfaces submerged in the marine environment, as previously suggested for *P. tunicata* strain D2 (Rao et al., 2005).

The analysis of 16S rRNA gene sequences from our global collection of *Pseudoalteromonas* confirms that phylogenetic analysis results in a number of clusters encompassing predominantly pigmented species or non-pigmented species (Ivanova et al., 2004). Strain S3431 was the single pigmented strain in the so-called non-pigmented clusters. Novel diversity might be represented in cluster III which consisted of strains with 98% or less 16S rRNA gene sequence similarity to *Pseudoalteromonas* type strains and formed a separate cluster in the phylogenetic analysis. However, these strains showed no antibacterial activity and no small molecule metabolites were detected. Such novel diversity could still represent untapped biotechnological potential, producing e.g. enzymes or peptides with biological activity, as known for other non-pigmented *Pseudoalteromonas* (Hoyoux et al., 2001;Violot et al., 2005).

Chemical profiling of the strains detected an array of secondary metabolites. In addition to complementing our analysis of 16S rRNA gene sequences, it also demonstrated that some

compounds (e.g. violacein, prodigiosin) were characteristic of a species, other compounds were produced by several species and we also detected intra-species clusters of different secondary metabolite profiles. In a broad sense, the clustering based on 16S rRNA gene similarity agreed with the groups resulting from the chemophylogenetic analysis. However, some compounds were produced by organisms of different species that then clustered together using the secondary metabolites as basis. The chemical analysis separated the 4 isolated *P. luteoviolacea* strains into two distinct sub-groups, showing intra-species chemical diversity. The *P. luteoviolacea* strains produced violacein and pentabromopseudilin which are active against gram-positive and gram-negative bacteria, and the anti-staphylococcal agent indolmycin (Hornemann et al., 1971;Hurdle et al., 2004). Violacein and pentabromopseudilin have previously been detected in *Pseudoalteromonas luteoviolacea* (Gauthier, 1976a;Laatsch and Pudleiner, 1989), but to our knowledge this is the first report of *Pseudoalteromonas* strains producing indolmycin (Månsson et al., 2010).

Within some species, all strains were consistently antibacterial. However, in others such activity did not appear to be a consistent trait of the species. For instance, strains of the 16S cluster VI (*P. phenolica*) were heterogeneous in their ability to inhibit *Vibrio* in our assays, while all but one strain in the homogeneus cluster VII had identical metabolite profiles and all were inhibitory. Even more obvious was the heterogeneous chemical profiles within the *P. rubra* strains. All except one strain shared a chemical marker prodigiosin and/or RT 15.99 min, but had major variations in 14 other compounds. This may in part be due to loss of ability to produce a compound. For instance, strain S2471 over time lost ability to produce the brominated indole (RT 13.78 min). Also, we note that the type strain DSM 6842 (ATCC 29570) did not in our culture produce prodigiosin which has been observed previously (Gauthier, 1976b;Gauthier and Flatau, 1976). The consistent bromoalterochromide production in the two species *P. piscicida* and *P. flavipulchra/maricaloris* (cluster VII) was expected (Speitling et al., 2007) and supported the high DNA sequence similarity between the two. This emphasizes the need to isolate and screen multiple strains from each species

when bioprospecting within the genus *Pseudoalteromonas*, as even the homogeneous cluster VII contains one strain with a metabolite profile that does not share a single compound with the other strains in this cluster.

Several of the 26 detected peaks were known substances, with a majority known as antibacterials. These included: violacein (Lichstein and Vandesand, 1945), two bromopseudilins (Lovell, 1966), two indolmycins (Werner, 1980), four quinolins (Wratten et al., 1977); and prodigiosin (Kalesperis et al., 1975). Due to its very low aqueous solubility violacein probably protects against predation rather than acts as a true antibiotic, and it has been shown to induce cell death in grazing organisms (Matz et al., 2008). Such compounds would be very beneficial for protection of a biofilm, which is likely how surface associated *Pseudoalteromonas* would grow. The 14 compounds that could not be identified were mainly not identified due to poor ionization in ESI^+ and ESI^- and/or several plausible candidates in Antibase2009. However, for chemotaxonomic studies identity of the compounds is not necessary as long as they can be unambiguously identified between samples (Frisvad et al., 2008).

Within cluster VIII (*P. ruthenica*) and cluster II (*aurantia/citrea*) we found examples where strains with highly similar 16S rRNA gene sequences (>99%) and with identical chemotaxonomy originated from geographically distinct locations. This latter observation is in agreement with studies on *Salinispora* biogeography and secondary metabolite production in which the authors show how strains of the marine bacterium *Salinispora arenicola* isolated from worldwide locations are highly related and produce identical patterns of secondary metabolites (Jensen and Mafnas, 2006;Jensen et al., 2007). In contrast, the *P. luteoviolacea* and *P. rubra* strains showed both local and global variations in their secondary metabolite profile, which one might speculate is due to adaptation to local specific niches.

In conclusion, we believe sampling from specific niches, e.g. biofilms on surfaces, to be of importance in discovery of novel secondary metabolites from the genus *Pseudoalteromonas*. While differences in metabolite patterns among species encourage isolation and screening of novel diversity, bioprospecting known *Pseudoalteromonas* species should not be ruled out. Investigation of multiple strains of one *Pseudoalteromonas* species can yield novel compounds due to intra-species variations within secondary metabolite profiles.

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Figure 1: Isolation sites of 101 *Pseudoalteromonas* strains with each color code representing one of the 8 clusters as indicated in Table 1. Each circle represents the isolation of one or more strains belonging to the given cluster. White = cluster I, yellow = cluster II, grey = cluster III, green = cluster IV, violet = cluster V, red = cluster VI, orange = cluster VII, pink = cluster VII, and blue = non-clustered strain.

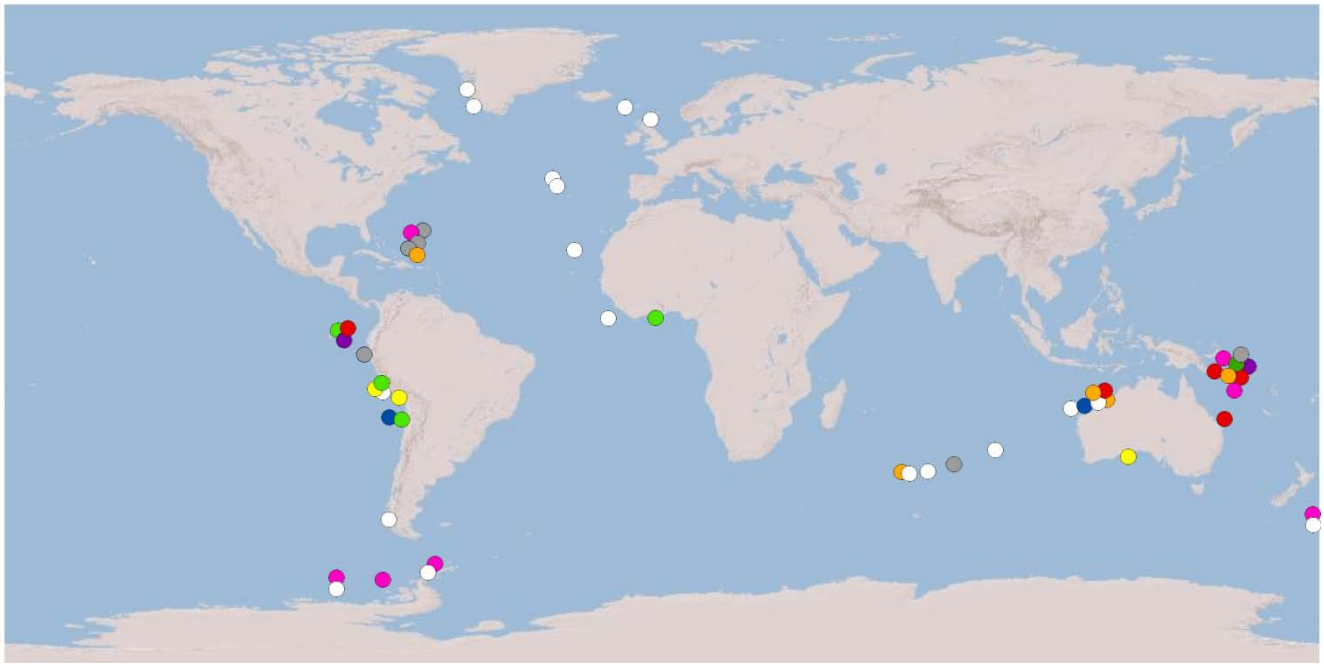


Figure 2: Phylogenetic tree based on 16S-rRNA gene sequences of the Galathea 3 *Pseudoalteromonas* strains. Sequences were aligned by MAFFT (default options) and the resulting alignment was used to generate a Neighbor Joining tree in the MEGA4 software package (default settings, 1000 bootstraps). *Salinispora arenicola* CNS-205, GenBank accession number CP000850, GeneID: 5705939 was used as outgroup. Clusters containing 2 or more non-type strain sequences were collapsed. The scale bar represents 0.02 amino acid substitutions per nucleotide position. ●: Nodes also occurring in minimum evolution and maximum likelihood trees.

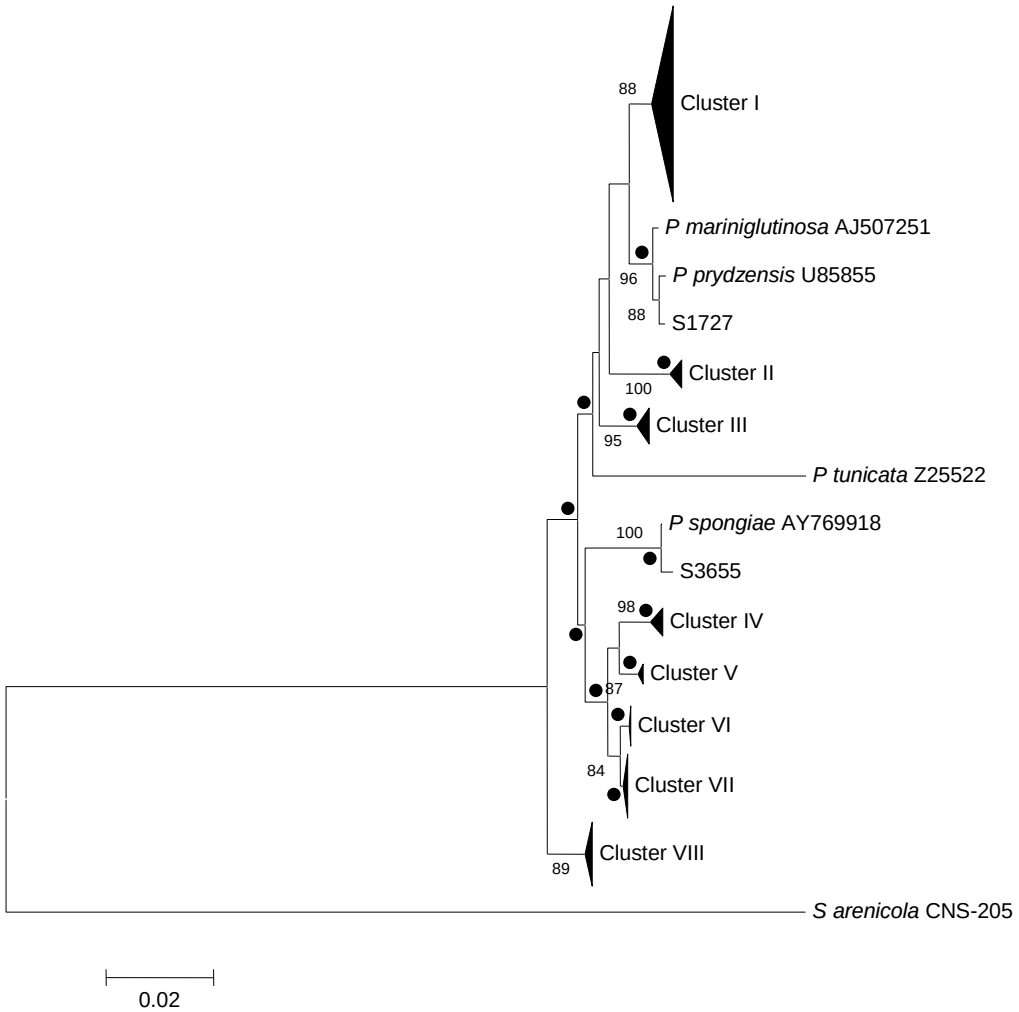


Figure 3: Dendrogram from cluster analysis of detected peaks from HPLC-UV/VIS detection of compounds in the ethyl acetate extracted broth and biomass. Data was processed in NTSYSpc 2.20q, with SAHN clustering by UPGMA and simple distance measurement.

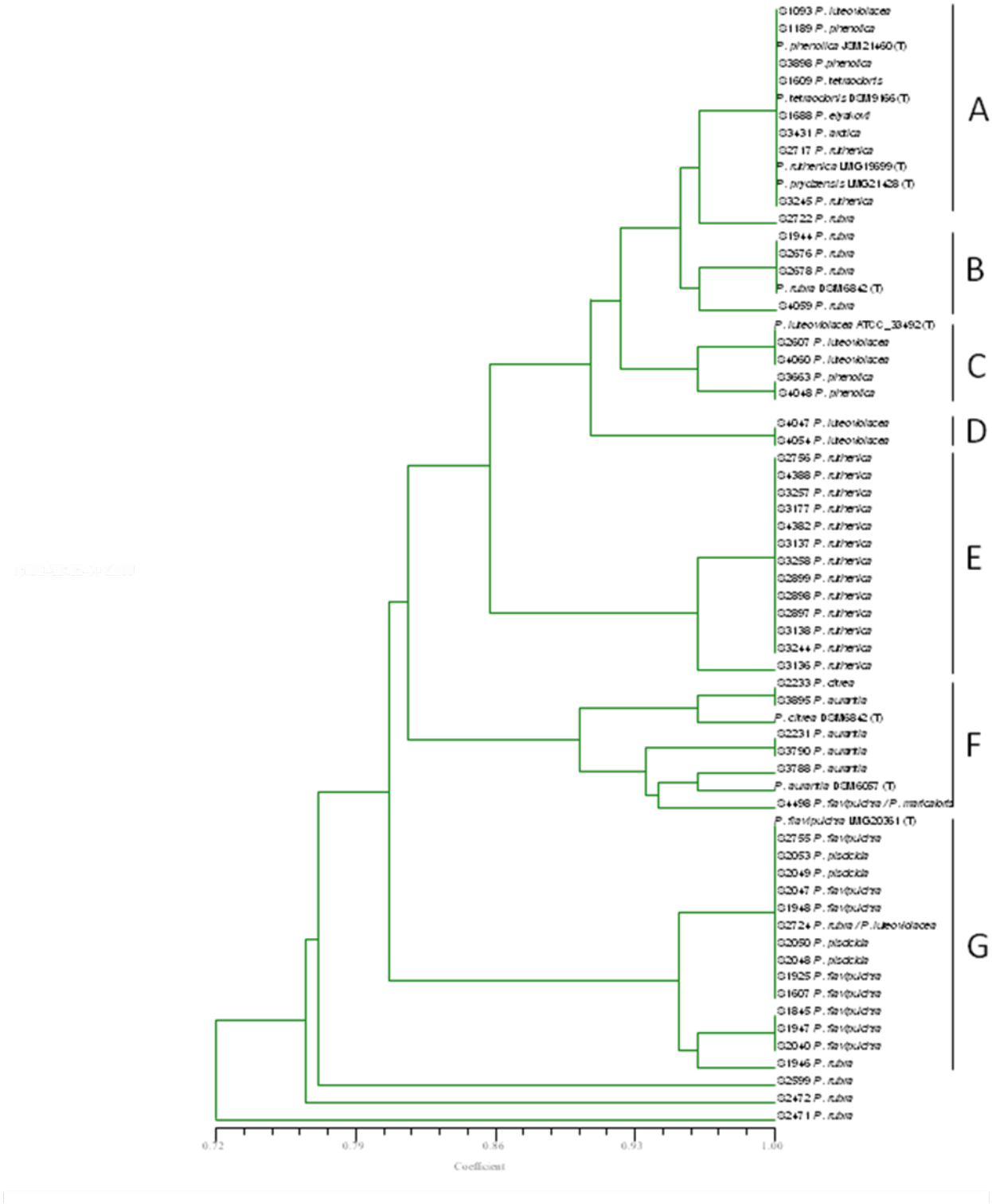


Figure 4: HPLC-UV/VIS profiles of the ethyl acetate extracted broth and biomass of four *P. luteoviolacea* with the tailing violacein peak at 14-15 min, and in the two upper traces pentabromopseudilin at 18 min in contrast to indolmycin at 11 min in the two lower traces.

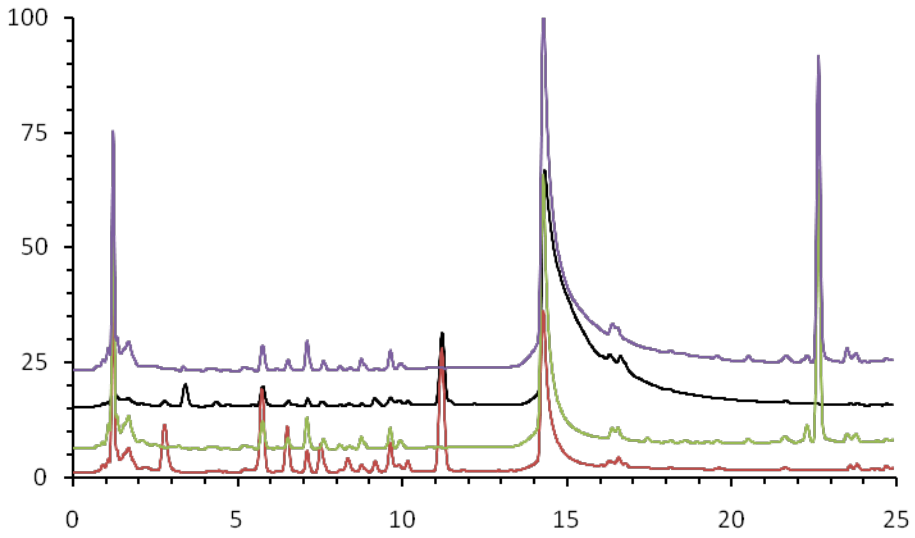


Table 1: Identity, antibacterial activity and pigmentation of *Pseudoalteromonas* strains from a global collection

16S-rRNA cluster	No. of strains		Related type strain[1]	No. of strains from		No. of strains inhibiting *Vibrio*		Inhibition of *Vibrio* by EtAc extracts
	Pigmented	Non-pigmented		Surface samples	Water samples	Pigmented	Non-pigmented	
I	1	37	*P. agarivorans*	16	22	0	0	0
II	5	0	*P. aurantia*	2	3	5	0	0
III	0	9	*P. prydzensis*	8	1	0	0	0
IV	3	3	*P. phenolica*	3	3	3	2	0
V	4	0	*P. luteoviolacea*	4	0	4	0	4
VI	9	0	*P. rubra*	9	0	9	0	0
VII	13	0	*P. flavipulchra*	13	0	13	0	0
VIII	15	0	*P. ruthenica*	14	1	15	0	15
S1727	0	1	*P. mariniglutinosa*	0	1	0	0	0
S3655	1	0	*P. spongiae*	1	0	0	0	0
Total	51	50		70	31	49	2	19

[1]: The type strain which the majority of the strains in the cluster were most closely related to

1

Table 2: Ethyl acetate extractable secondary metabolites produced by pigmented *Pseudoalteromonas* strains

Compound	MI (Da)[1]	UV-max data	RT (min)	Code
Indolmycin[2]	257		11.21	A
2-Pentyl-4-quinolinol[3]	215		12.31	B
Novel mono brominated indole	280		13.78	C
Violacein[2]	343		14.29	D
Unidentified	244	212nm (100%), 250nm (48%)	15.21	E
2-n-Heptyl-(1H)-quinolin-4-one[3]	243		15.47	F
Unidentified	NI	228nm (45%), 308 nm (100%)	15.70	G
Unidentified	386	< 200 nm	15.78	H
Unidentified	316	286nm (100%)	15.80	I
Unidentified	NI[4]	< 200 nm	15.81	J
Unidentified	NI	228nm (45%), 308nm (100%)	15.98	K
Unindentified	676	< 200nm	15.99	L
Prodigiosin[2]	323		16.00	M
Unidentified	NI	< 200nm	16.26	N
Unidentified	NI	228nm (45%), 308nm (100%)	16.30	O
Bromoalterochromide A[3]	843		16.49	P
Novel bromoalterochromide, 2 bromine	921		16.60	Q
Novel bromoalterochromide, 1 bromine	857		17.10	R
Unidentified	333	310nm (100%)	17.20	S
2-n-Nonyl-(1H)-quinolin-4-one[3]	271		17.96	T
Nonyl-quinolin-one analogue[3]	271		18.28	U
Unidentified	315	362nm (100%)	18.90	V
Unidentified	244	218nm (100%), 280nm (82%)	19.42	W
Unidentified	NI	218nm (100%), 288nm (82%)	19.78	X
Unidentified	NI	250nm (100), 280nm (86)	19.92	Y
Pentabromopseudilin[3]	549		22.65	Z

[1] Mono-isotopic mass

[2] validated reference standard used for verification.

[3] Accurate mass and UV data fits data from Antibase2009

[4] NI No ionization or MI could not be assigned using ESI^+ and ESI^-

Table 3. Secondary metabolites produced by *Pseudoalteromonas* species clustered by 16S rRNA gene similarity. Identification of peak by capital letter in table 2

		Peak at retention time present in *Pseudoalteromonas* strain/organism																												
16S cluster	# strains	none	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T	U	V	W	X	Y	Z
I	38	x																										
P.tetraodonis DSM9166		x																										
P.prydzensis LMG21428		x																										
II	1			x				x														x						
	2			x				x														x	x					
	1			x				x											x				x					
	1			x				x											x				x					
P. aurantia DSM6057				x																		x						
P.citrea DSM8771				x				x											x									
III	9	x																										
IV	3	x																										
	1																	x	x	x								
	2																											x
P.phenolica DSM21460		x																										
V	2					x																						x
	2		x			x																						
P.luteoviolacea ATCC33492						x																						x
VI	1													x														
	1														x			x	x	x							x	
	1				x		x				x														x	x		
	1											x			x						x		x					
	1								x					x	x	x													
	2													x															
	1														x														
	1													x	x														
P.rubra DSM6842														x															
VII	9																	x	x	x									
	3																	x	x	x								x	
	1			x				x																					
P.flavipulchra LMG20361																		x	x	x									
VIII	2	x																											
	13									x			x				x												
P.ruthenica LMG19699		x																											
S1727	1	x																											
S3655	1	x																											

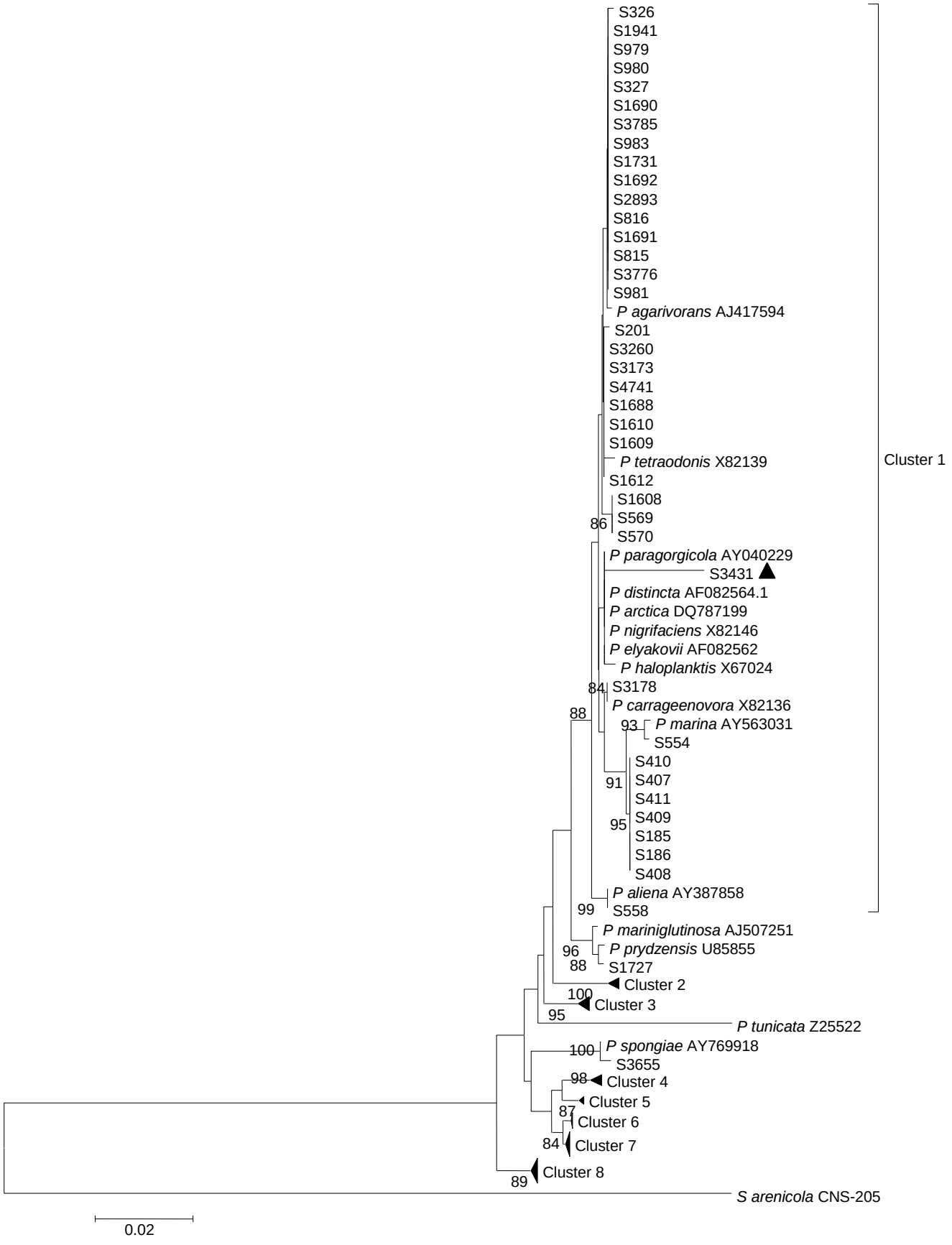
Supplementary material

Figure and table legends

Supplementary Figure 1: The phylogenetic tree also shown in figure 2, here with cluster I not collapsed to provide an overview of the large number of strains contained therein. The filled black triangle marks strain S3431, which in the BLAST analysis showed a low (97%) level of similarity to *Pseudoalteromonas* type strains.

Supplementary Table 1: Origin and anti-bacterial activity of *Pseudoalteromonas* strains included in this study. The cluster designation refers to figure 1. Sequence identity was determined using the BLAST algorithm. Where two hits are listed they scored equally in BLAST analysis.

Supplementary Figure 1: The phylogenetic tree also shown in figure 2, here with cluster I not collapsed to provide an overview of the large number of strains contained therein. The filled black triangle marks strain S3431, which in the BLAST analysis showed a low (97%) level of similarity to *Pseudoalteromonas* type strains.



Supplementary Table 1: Origin and anti-bacterial activity of *Psedoalteromonas* strains included in this study. The cluster designation refers to figure 1. Sequence identity was determined using the BLAST algorithm. Where two hits are listed they scored equally in the BLAST analysis.

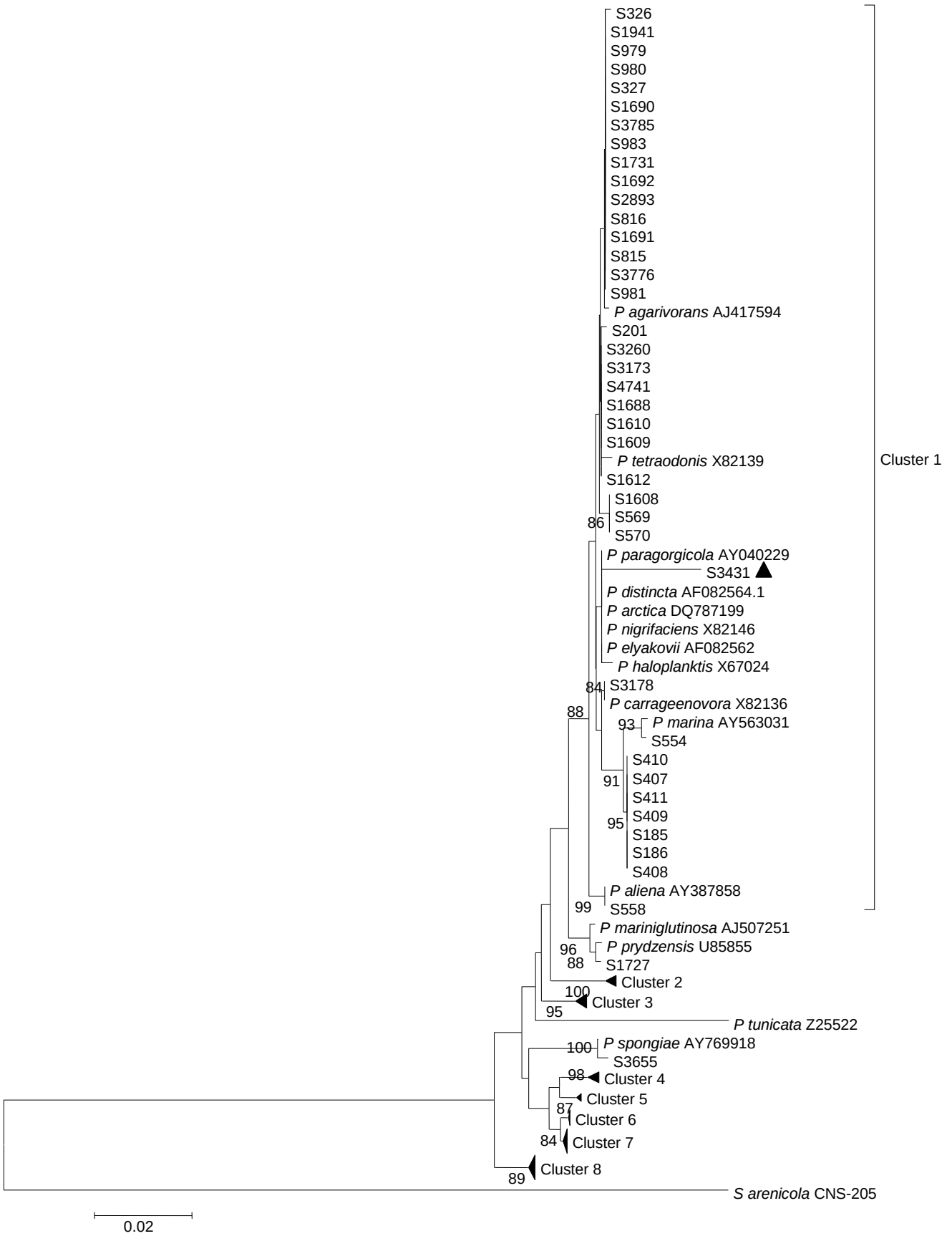
Strain	GenBank acc. #	Latitude +=N	Longitude +=E	Water or surface	BLAST type strain match	% BLAST ident.	Cluster 16S	Cluster Chem.	Pigment	Inhibition of *V. anguillarum* Spot-assay	Sup	EtAc ex
S185	FJ457121	62.03815	-9.99592	W	*P. arctica*	98	1	A	-	-	-	-
S186	FJ457122	62.03815	-9.99592	W	*P. distinct*	98	1	A	-	-	-	-
S187	FJ457123	62.03815	-9.99592	W	*P. prydzensis*	97	3	A	-	-	-	-
S201	FJ457124	62.03815	-9.99592	W	*P. agarivorans* *P. distincta*	99	1	A	-	-	-	-
S326	FJ457125	62.2603	-51.6539	S	*P. agarivorans*	99	1	A	-	-	-	-
S327	FJ457126	62.2603	-51.6539	S	*P. agarivorans*	99	1	A	-	-	-	-
S407	FJ457127	66.90525	-53.2894	W	*P. nigrifaciens*	98	1	A	-	-	-	-
S408	FJ457128	66.90525	-53.2894	W	*P. nigrifaciens*	98	1	A	-	-	-	-
S409	FJ457129	66.90525	-53.2894	W	*P. nigrifaciens*	98	1	A	-	-	-	-
S410	FJ457130	66.90525	-53.2894	W	*P. nigrifaciens*	98	1	A	-	-	-	-
S411	FJ457131	66.90525	-53.2894	W	*P. nigrifaciens*	98	1	A	-	-	-	-
S554	FJ457133	42.6047	-29.9597	S	*P. marina*	99	1	A	-	-	-	-
S558	FJ457134	42.6047	-29.9597	S	*P. aliena*	99	1	A	-	-	-	-
S569	FJ457136	40.67227	-28.8374	W	*P. tetraodonis*	99	1	A	-	-	-	-
S570	FJ457137	40.67227	-28.8374	W	*P. tetraodonis*	99	1	A	-	-	-	-
S815	FJ457139	23.07925	-24.0523	W	*P. agarivorans*	99	1	A	-	-	-	-
S816	FJ457140	23.07925	-24.0523	W	*P. agarivorans*	99	1	A	-	-	-	-
S979	FJ457141	23.07918	-24.0542	W	*P. agarivorans*	99	1	A	-	-	-	-
S980	FJ457142	23.07918	-24.0542	W	*P. agarivorans*	99	1	A	-	-	-	-
S981	FJ457143	23.07918	-24.0542	W	*P. agarivorans*	99	1	A	-	-	-	-
S983	FJ457144	4.568133	-14.7097	W	*P. agarivorans*	99	1	A	-	-	-	-
S1093	FJ457145	4.570467	-1.72975	W	*P. luteoviolacea*	98	4	A	-	+	+	-
S1189	FJ457146	4.570467	-1.72975	W	*P. phenolica*	98	4	A	Brown	+	+	-

S1607	FJ457149	-38.407	66.3738	S	*P. flavipulchra*	99	7	G	Yellow	+	-	-
S1608	FJ457150	-38.407	66.3738	S	*P. agarivorans*	99	1	A	-	-	-	-
S1609	FJ457151	-38.407	66.3738	S	*P. tetraodonis*	100	1	A	-	-	-	-
S1610	FJ457152	-38.407	66.3738	S	*P. agarivorans* *P. paragorgicola*	99	1	A	-	-	-	-
S1612	FJ457153	-37.2168	72.7091	S	*P. agarivorans* *P. paragorgicola*	99	1	A	-	-	-	-
S1649	FJ457154	-35.3413	79.9641	S	*P. prydzensis*	98	3	A	-	-	-	-
S1650	FJ457155	-35.3413	79.9641	S	*P. prydzensis*	98	3	A	-	-	-	-
S1688	FJ457157	-31.4061	91.17758	W	*P. elyakovii*	99	1	A	-	-	-	-
S1690	FJ457158	-31.4061	91.17758	W	*P. agarivorans*	99	1	A	-	-	-	-
S1691	FJ457159	-31.4061	91.17758	W	*P. agarivorans*	99	1	A	-	-	-	-
S1692	FJ457160	-31.4061	91.17758	W	*P. agarivorans*	99	1	A	-	-	-	-
S1727	FJ457161	-19.7461	114.8573	W	*P. mariniglutinosa*	99	-	A	-	-	-	-
S1731	FJ457162	-19.7461	114.8573	W	*P. agarivorans*	99	1	A	-	-	-	-
S1845	FJ457163	-17.7746	121.8656	S	*P. flavipulchra*	99	7	G	Yellow	+	-	-
S1925	FJ457165	-17.0038	120.7788	S	*P. flavipulchra*	99	7	G	Yellow	+	-	-
S1941	FJ457166	-17.2706	121.1293	W	*P. agarivorans*	99	1	A	-	-	-	-
S1944	FJ457168	-17.0038	120.7788	S	*P. rubra*	99	6	B	Red	+	+	-
S1946	FJ457169	-17.0038	120.7788	S	*P. rubra*	99	6	G	Red	+	+	-
S1947	FJ457170	-17.0038	120.7788	S	*P. flavipulchra*	99	7	G	Yellow	+	-	-
S1948	FJ457171	-17.0038	120.7788	S	*P. flavipulchra*	99	7	G	Yellow	+	-	-
S2040	FJ457173	-16.0604	119.3541	S	*P. flavipulchra*	99	7	G	Yellow	+	-	-
S2047	FJ457174	-16.0604	119.3541	S	*P. flavipulchra*	99	7	G	Yellow	+	-	-
S2048	FJ457175	-16.0604	119.3541	S	*P. piscicida*	99	7	G	Yellow	+	-	-
S2049	FJ457176	-16.0604	119.3541	S	*P. piscicida*	99	7	G	Yellow	+	-	-
S2050	FJ457177	-16.0604	119.3541	S	*P. piscicida*	99	7	G	Yellow	+	-	-
S2053	FJ457179	-16.0604	119.3541	S	*P. piscicida*	99	7	G	Yellow	+	-	-
S2231	FJ457180	-33.3267	127.6573	S	*P. aurantia* *P. citrea*	99	2	F	Pale yellow	+	+	-
S2233	FJ457181	-33.3267	127.6573	S	*P. citrea*	99	2	F	Pale yellow	+	+	-
S2471	FJ457184	-22.9636	153.9486	S	*P. rubra*	99	6	-	Red	+	+	-

S2472	FJ457185	-22.9636	153.9486	S	*P. rubra*	99	6	-	Red	+	+	-
S2599	FJ457186	-10.3454	157.7956	S	*P. rubra*	99	6	-	Red	+	+	-
S2607	FJ457187	-10.3454	157.7956	S	*P. luteoviolacea*	99	5	C	Purple	+	+	+
S2676	FJ457188	-8.0692	155.8781	S	*P. rubra*	99	6	B	Red	+	+	-
S2678	FJ457189	-8.0692	155.8781	S	*P. rubra*	99	6	B	Red	+	+	-
S2717	FJ457190	-7.8244	156.0689	W	*P. ruthenica*	99	8	A	Brown	+	-	+
S2721	FJ457191	-8.1005	156.8451	S	*P. prydzensis*	97	3	A	-	-	-	-
S2722	FJ457192	-8.1005	156.8451	S	*P. rubra*	99	6	-	Red	+	+	-
S2724	FJ457193	-8.1005	156.8451	S	*P. rubra* *P. luteoviolacea*	97	4	G	Brown	+	-	-
S2755	FJ457196	-9.108	156.8595	S	*P. flavipulchra*	99	7	G	Yellow	+	-	-
S2756	FJ457197	-15.2329	156.665	S	*P. ruthenica*	99	8	E	Brown	+	-	+
S2893	FJ457198	-48.915	178.1056	S	*P. agarivorans*	99	1	A	-	-	-	-
S2897	FJ457199	-48.915	178.1056	S	*P. ruthenica*	99	8	E	Brown	+	-	+
S2898	FJ457200	-48.915	178.1056	S	*P. ruthenica*	99	8	E	Brown	+	-	+
S2899	FJ457201	-48.915	178.1056	S	*P. ruthenica*	99	8	E	Brown	+	-	+
S3136	FJ457202	-66.7706	-76.4383	S	*P. ruthenica*	99	8	E	Brown	+	-	+
S3137	FJ457203	-66.7706	-76.4383	S	*P. ruthenica*	99	8	E	Brown	+	-	+
S3138	FJ457204	-66.7706	-76.4383	S	*P. ruthenica*	99	8	E	Brown	+	-	+
S3173	FJ457205	-67.4876	-89.1285	S	*P. tetraodonis*	98	1	A	-	-	-	-
S3177	FJ457206	-67.4876	-89.1285	S	*P. ruthenica*	99	8	E	Brown	+	-	+
S3178	FJ457207	-67.4876	-89.1285	S	*P. carrageenovora*	99	1	A	-	-	-	-
S3244	FJ457208	-67.4876	-89.1285	S	*P. ruthenica*	99	8	E	Brown	+	-	+
S3245	FJ457209	-67.4876	-89.1285	S	*P. ruthenica*	99	8	A	Brown	+	-	+
S3257	FJ457210	-64.0947	-62.8228	S	*P. ruthenica*	99	8	E	Brown	+	-	+
S3258	FJ457211	-64.0947	-62.8228	S	*P. ruthenica*	99	8	E	Brown	+	-	+
S3260	FJ457212	-64.0947	-62.8228	S	*P. agarivorans*	99	1	A	-	-	-	-
S3431	FJ457214	-50.4498	-74.8912	S	*P. arctica*	97	1	A	Black	-	-	-
S3655	FJ457216	-20.0568	-70.755	S	*P. spongiae*	99	-	A	Orange	-	-	-
S3663	FJ457217	-20.0568	-70.755	S	*P. phenolica*	97	4	C	-	+	-	-
S3776	FJ457222	-14.2295	-76.6073	S	*P. agarivorans*	99	1	A	-	-	-	-
S3785	FJ457223	-14.2295	-76.6073	S	*P. agarivorans*	99	1	A	-	-	-	-

S3788	FJ457224	-17.0857	-72.419	W	*P. aurantia*	98	2	F	Pale yellow	+	+	-
S3790	FJ457225	-17.0857	-72.419	W	*P. aurantia*	98	2	F	Pale yellow	+	+	-
S3895	FJ457226	-14.1631	-77.4286	W	*P. aurantia*	98	2	F	Pale yellow	+	+	-
S3898	FJ457228	-13.818	-76.7648	W	*P. phenolica*	97	4	A	-	-	-	-
S3944	FJ457229	-5.3492	-81.4284	S	*P. mariniglutinosa*	98	3	A	-	-	-	-
S4047	FJ457230	2.9817	-86.6892	S	*P. luteoviolacea*	98	5	D	Purple	+	+	+
S4048	FJ457231	2.9817	-86.6892	S	*P. phenolica*	98	4	C	Brown	+	-	-
S4054	FJ457234	2.9817	-86.6892	S	*P. luteoviolacea*	99	5	D	Purple	+	+	+
S4059	FJ457237	2.9817	-86.6892	S	*P. rubra*	99	6	B	Red	+	+	-
S4060	FJ457238	2.9817	-86.6892	S	*P. luteoviolacea*	98	5	C	Purple	+	+	+
S4382	FJ457239	26.5042	-66.9964	S	*P. ruthenica*	99	8	E	Brown	+	-	+
S4388	FJ457240	26.5042	-66.9964	S	*P. ruthenica*	99	8	E	Brown	+	-	+
S4389	FJ457241	26.5042	-66.9964	S	*P. mariniglutinosa*	97	3	A	-	-	-	-
S4488	FJ457243	24.9963	-67.0246	S	*P. prydzensis*	97	3	A	-	-	-	-
S4491	FJ457244	24.9963	-67.0246	S	*P. prydzensis*	97	3	A	-	-	-	-
S4492	FJ457245	24.9963	-67.0246	S	*P. mariniglutinosa*	98	3	A	-	-	-	-
S4498	FJ457247	24.9963	-67.0246	S	*P. flavipulchra* *P. maricaloris*	99	7	F	-	+	-	-
S4741	FJ457248	58.8041	-3.0564	S	*P. elyakovii*	99	1	A	-	-	-	-

Supplementary figure S1: The phylogenetic tree also shown in figure 2, here with cluster I not collapsed to provide an overview of the large number of strains contained therein. The filled black triangle marks strain S3431, which in the BLAST analysis showed a low (97%) level of similarity to *Pseudoalteromonas* type strains.


S326
S1941
S979
S980
S327
S1690
S3785
S983
S1731
S1692
S2893
S816
S1691
S815
S3776
S981
P agarivorans AJ417594
S201
S3260
S3173
S4741
S1688
S1610
S1609
P tetraodonis X82139
S1612
S1608
S569
S570
P paragorgicola AY040229
S3431
P distincta AF082564.1
P arctica DQ787199
P nigrifaciens X82146
P elyakovii AF082562
P haloplanktis X67024
S3178
P carrageenovora X82136
P marina AY563031
S554
S410
S407
S411
S409
S185
S186
S408
P aliena AY387858
S558
P mariniglutinosa AJ507251
P prydzensis U85855
S1727
Cluster 2
Cluster 3
P tunicata Z25522
P spongiae AY769918
S3655
Cluster 4
Cluster 5
Cluster 6
Cluster 7
Cluster 8
S arenicola CNS-205
Cluster 1
86
84
88
93
91
95
99
96
88
100
95
100
98
87
84
89
0.02